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Investigation of Glyphosate-Resistant Horseweed (*Conyza canadensis* L. Cronq.) In Tennessee

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To the Graduate Council:

I am submitting herewith a dissertation written by Christopher Lynn Main entitled "Investigation of Glyphosate-Resistant Horseweed (*Conyza canadensis* L. Cronq.) In Tennessee." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

Thomas C. Mueller, Major Professor

We have read this dissertation and recommend its acceptance:

Robert M. Hayes, Vincent R. Pantalone, C. Neal Stewart, John B. Wilkerson

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor and Dean of
Graduate Studies

INVESTIGATION OF GLYPHOSATE-RESISTANT HORSEWEED (*Conyza canadensis* L. Cronq.) IN TENNESSEE

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee

Christopher Lynn Main
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To God, by whose grace all things are possible.

ABSTRACT

This research had multiple objectives: 1) confirm resistance to glyphosate in a regional horseweed [*Conyza canadensis* (L.) Cronq.] population; 2) determine the location of glyphosate-resistant horseweed in the lower mid-west and mid-south states; 3) determine the level of glyphosate-resistance in horseweed biotypes from Tennessee; 4) examine the accumulation of shikimate in both glyphosate-resistant (GR) and glyphosate-susceptible (GS) horseweed plants.

The analytical procedure to determine shikimate used extraction with 1 M HCl for 16 hr followed by liquid chromatography using photo-diode array detection, and shikimate recoveries were ≥82%. Glyphosate application of either 0.84 kg ae/ha (the standard application rate) and 3.8 kg ae/ha to susceptible plants caused complete plant death to susceptible plants. Glyphosate applied at 3.8 kg ae/ha to putative resistant populations caused < 15 % growth reduction as determined by visual evaluations, and fresh weights of these resistant plants 17 days after glyphosate treatment (DAT) were reduced an average of 45 % in one population (susceptible), and were not affected in a different population (denoted resistant). This direct comparison conclusively confirms that horseweed plants collected in western Tennessee in 2002 were resistant to four times the normal application dosage of glyphosate. The GR horseweed biotypes still exhibited some herbicidal effects from the glyphosate, such as yellowing in the most actively growing, apical shoot meristems. The yellowing in the shoot apices was transitory, older leaf tissue became necrotic, and the plants recovered from this damage to continue growth. Shikimate concentrations in all untreated horseweed plants were less than 100

μg/g, which was significantly less than all plants which had been treated with 0.84 kg ae/ha of glyphosate. Unexpectedly, shikimate accumulated (>1000 μg/g) in both resistant populations and in the GS population. However, there were differences in shikimate accumulation patterns between resistant and susceptible horseweed biotypes. Shikimate concentrations in resistant populations declined about 40 % from 2 to 4 DAT, while shikimate concentrations in the susceptible horseweed plants increased about 35 % from 2 to 4 DAT. The confirmed resistance of a widespread weed implies that alternative control strategies for GR horseweed will be needed in those no tillage production systems were it commonly occurs.

Horseweed seed were collected in Illinois, Indiana, Kentucky, Mississippi, Missouri and Ohio to determine susceptibility of different horseweed biotypes to glyphosate. Horseweed resistant to glyphosate were found in Mississippi, Ohio, and western Tennessee. In a separate experiment examining Tennessee biotypes, a dose response curve demonstrated that four times as much glyphosate was needed to achieve a 50% fresh weight reduction (GR_{50}) in resistant biotypes when compared to a susceptible biotype. Resistant biotypes from Tennessee displayed a GR_{50} of 1.6 kg/ha, as compared to a GR_{50} of 0.4 kg/ha in a susceptible horseweed population.

A more comprehensive analysis of the response of shikimic acid levels in shoot and root tissue of GS and GR horseweed biotypes was conducted. Both horseweed biotypes displayed an increase in shikimic acid indicating that 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) remained sensitive to glyphosate. Shikimic acid levels in both shoots and roots of GS horseweed displayed a increasing sigmoidal response to

glyphosate, while in GR horseweed shikimic acid levels displayed an increasing log normal peak response with a maximum concentration occurring around 72 hours after treatment (HAT) in both shoot and root tissue. Shikimic acid concentration in GR horseweed began to decrease between 72 and 96 HAT indicating that the shikimic acid pathway resumed at least partial function in the presence of glyphosate. At 168 HAT shikimic acid levels in GS horseweed shoot tissue displayed a 6:1 increase and a 3:1 increase in root tissue when compared to GR horseweed. This ratio corresponds to previously observed differences in whole plant sensitivity to glyphosate for GS and GR horseweed.

These results imply that horseweed resistance to glyphosate is not due to a change in the site of herbicide action. The mechanism of resistance appears to be similar to GR ryegrass and different from glyphosate tolerant crops. GR horseweed biotypes required four times more glyphosate to achieve 50% growth reduction when compared to GS horseweed biotypes. Shikimic acid decrease over time could be due to the presence of three isoforms of EPSPS and possible glyphosate induced amplification of the genes coding for EPSPS. Changes in EPSPS may allow the shikimate acid pathway to operate depleting the shikimic acid pool, leading to continued plant growth.

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PART I.

INTRODUCTION

Horseweed [*Conyza canadensis* (L.) Cronq.], [syn. *Erigeron canadensis* (L.)]is in the Asteraceae family and commonly grows throughout North America (1). Horseweed (also referred to as Canada fleabane or mare's-tail) has been demonstrated to be a problem in conservation tillage production systems in cotton (*Gossypium hirsutum* L.) in Alabama (2), in grain sorghum [*Sorghum bicolor* (L.) Moench] in Georgia (3), in corn (*Zea mays* L.) in Wisconsin (4), and soybean [Glycine max (L.) Merr.] and corn in Iowa and Minnesota (5), in fallow periods in the southern Great Plains (6), and in the production of container-grown ornamentals (7). Seed dispersal is the main mechanism of horseweed spread. Horseweed plants are capable of producing over 200,000 small, wind-dispersed seeds per plant in late summer (1). Horseweed, like many other weeds, displays a high degree of plasticity in plant size and reproductive capacity (8).

Horseweed is an annual plant that has traditionally been thought to germinate in late summer or fall, overwinter as a small rosette, bolt the following spring, and then produce seed in summer (2,5,8,9). However, Regeher and Bazzaz (8) indicate horseweed germination can also occur in spring. Tillage of the soil for establishment of summer annual crops disrupts the life cycle of winter annuals such as horseweed. Research has demonstrated that fall or spring tillage controls horseweed and other winter annual weeds (2). No-tillage farming practices create an environment where soil is not disturbed and herbicides replace tillage for weed control prior to seeding an annual crop (10). Winter annual plants which have germinated during fall and survived the winter have a competitive advantage over spring-germinated plants for space, water, light, and nutrients; there by suppressing the growth of summer annuals. Regeher and Bazzaz (8)

consider horseweed a successional winter annual that can rapidly infest abandoned fields. Holm et. al (9) indicates that horseweed is adapted to periodically plant-free, undisturbed soil, and was found to become established with an absence of tillage in crop production (2, 4). The opportunistic nature of horseweed establishment in undisturbed areas makes it well suited for infesting agricultural fields and surrounding areas.

Horseweed has been reported in Delaware (11) and western Tennessee (12) that is not completely controlled by normal applications of glyphosate. This decreased control is markedly different from what is expected since historically glyphosate provided essentially complete control of horseweed (13, 14, 15). Resistance is not the same as a weed shift, where different species that were never controlled or were poorly controlled by glyphosate increase in relative abundance in that environmental setting. Previous research has indicated the propensity of horseweed to develop resistance to herbicides. Populations of horseweed resistant to the herbicide paraquat were found in Ontario, Canada (16). These paraquat-resistant populations required doses > 25 times higher than susceptible populations for equivalent control. Horseweed resistant to paraquat (17) and triazines (18) was also documented from collections in Hungary. Glyphosate resistance in horseweed represents a change at the physiological level.

Naturally-occurring evolved glyphosate resistance has occurred in perennial ryegrass (*Lolium perenne L.*), fine fescue (*Festuca* spp.) varieties (19, 20), and biotypes of birdsfoot trefoil (*Lotus corniculatus L.*) (21). Glyphosate resistance has also been reported in rigid ryegrass in Australia (22), goosegrass (*Elusine indica*) in Malaysia (23), and annual ryegrass (*Lolium multiflorum*) in Chile (24).

Glyphosate has a unique mode of action in plants (25, 26, 27). It inhibits aromatic amino acid biosynthesis, leading to blockage of protein and secondary metabolite production (25, 26). It works by competitive inhibition of the enzyme 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), which catalyzes an essential step in the aromatic amino acid biosynthetic pathway. EPSPS catalyzes the reaction of shikimate-3-phosphate (S3P) and phosphenolpyruvate (PEP) to yield 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate. EPSP is a precursor to chorismate formation, the base molecule for all aromatic amino acid formation. Measurement of shikimic acid accumulation in response to glyphosate inhibition of EPSPS is a rapid and accurate assay to quantify glyphosate-induced damage in sensitive plants (27).

Current glyphosate-resistant crop technology in soybean utilizes an EPSPS gene from *Agrobacterium spp.* strain CP4 (28, 29, 30). This CP4 gene synthesizes CP4 EPSPS in transformed plants which functions to catalyze the S3P - PEP reaction when naturally-occurring EPSPS is inhibited by glyphosate (19). Furthermore, an additional strategy to create some glyphosate-resistant crops includes adding a transgene for glyphosate degradation (30).

The first reported occurrence of glyphosate-resistant horseweed in North America was in Delaware in 2000 (11). No-tillage corn and soybean production has been widely adopted in the mid-Atlantic region, which has favored the establishment of horseweed. Within three years of using only glyphosate for weed control in continuous cropping of glyphosate-resistant soybeans, glyphosate failed to control horseweed in some fields (11). Seedlings originating from seed of one horseweed population in Delaware were grown in

the greenhouse, where they were ten-fold more resistant to glyphosate than a susceptible population. There were no reported differences in resistance level with different formulations of glyphosate.

A similar scenario occurred in western Tennessee with the introduction of glyphosate resistant soybeans and cotton in the mid to late 1990's. The use of glyphosate-resistant crops for weed control is common in no-tillage farming practices, which reduce erosion of the areas loess soil deposits by up to 90% (31). In a no-till production system, herbicides are the primary means of weed control due to the lack of soil disturbance. Continual use of glyphosate for preplant weed control and postemergence weed control in glyphosate resistant crops has led to the exclusive use of glyphosate on many crop acres.

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PART II

SHIKIMATE ACCUMULATES IN BOTH GLYPHOSATE-SENSITIVE AND GLYPHOSATE-RESISTANT HORSEWEED [*Conyza canadensis* (L.) Cronq.][1]

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ABSTRACT

Horseweed (*Conyza canadensis*) is a cosmopolitan weed that commonly grows throughout North America. Horseweed that is not completely controlled by normal applications of glyphosate has been reported in western Tennessee. This research had three objectives: 1) develop and validate an analytical procedure for the quantitative determination of shikimate, an important indicator of glyphosate activity in plants; 2) confirm resistance to glyphosate in a horseweed population; 3) examine the accumulation of shikimate in both glyphosate-resistant and glyphosate-susceptible horseweed plants. The analytical procedure to determine shikimate used extraction with 1 M HCl for 24 hr followed by liquid chromatography using photo diode array detection, and shikimate recoveries were ≥82%. Glyphosate application of either 0.84 kg ae/ha (the standard application rate) and 3.8 kg ae/ha to susceptible plants caused complete plant death. The same glyphosate applications to putative resistant populations caused less than 15 % growth reduction as determined by visual evaluations, and fresh weights of these resistant plants 17 days after glyphosate treatment (DAT) were reduced an average of 45 % in one population, and were not affected in a different population. This direct comparison conclusively confirms that horseweed plants collected in western Tennessee in 2002 are resistant to four times the normal application dosage of glyphosate. The glyphosate-resistant horseweed biotypes still exhibited some herbicidal effects from the glyphosate, such as yellowing in the most actively growing, apical shoot meristems. The yellowing in the shoot apices was transitory, and the plants recovered from this damage.

Shikimate concentrations in all untreated horseweed plants were less than 100 μg/g, which was significantly less than all plants which had been treated with 0.84 kg ae/ha of glyphosate. Unexpectedly, shikimate accumulated (>1000 μg/g) in both resistant populations and in the susceptible population. However, there were differences in shikimate accumulation patterns between resistant and susceptible horseweed biotypes. Shikimate concentrations in resistant populations declined about 40 % from 2 to 4 DAT, while shikimate concentrations in the susceptible horseweed plants increased about 35 % from 2 to 4 DAT. The confirmed resistance of a widespread weed implies that alternative control strategies for glyphosate-resistant horseweed will be needed in those no tillage production systems were it commonly occurs.

Keywords. *shikimate, HPLC, weed-resistance, glyphosate, EPSPS, herbicide resistance.*

INTRODUCTION

A common perspective in the late 1990s was that weed resistance to the herbicide glyphosate was not probable (1). This was believed because the complex manipulations of the target EPSPS enzyme required for the development of glyphosate-resistant crops were not expected to be duplicated in nature to evolve glyphosate-resistant weeds. This assessment is no longer true.

Horseweed (*Conyza canadensis* (L.) Cronq.) (also referred to as Canada fleabane or mare's-tail) is an annual plant, native to North America (2). Horseweed is a substantial problem in conservation tillage production systems in cotton (*Gossypium hirsutum* L.) in

Alabama (3), in grain sorghum [*Sorghum bicolor* (L.) Moench] in Georgia (4), in corn (*Zea mays* L.) in Wisconsin (5), in soybean [*Glycine max* (L.) Merr.]and corn in Iowa and Minnesota (6), in fallow periods in the southern Great Plains (7), and in the production of container-grown ornamentals (8). Horseweed is present throughout the North American continent. Large numbers of small, wind-dispersed seeds are produced in late summer (2). It serves as a wild host of the tarnished plant bug, and of aster yellows, a mycoplasma disease transmitted by the aster leaf hopper.

The first reported occurrence of glyphosate-resistant horseweed in North America was in Delaware in 2000 (9). No till corn and soybean production has been widely adopted in the mid-Atlantic region, which has favored the establishment of horseweed. Within three years of using only glyphosate for weed control in continuous cropping of glyphosate resistant soybeans, glyphosate failed to control horseweed in some fields. Seedlings originating from seed of one horseweed population in Delaware were grown in the greenhouse and exhibited greater than ten-fold resistance to glyphosate compared with a susceptible population. There were no reported differences in tolerance between different salts of glyphosate. Historically, glyphosate provided essentially complete control of horseweed (10, 11, 12), so this decreased control is markedly different. This weed resistance phenomenon differs from a herbicide-induced weed shift, where species that were never controlled or were poorly controlled by glyphosate increase in relative abundance in that environmental setting. This glyphosate resistance present in these

horseweed populations represents a change at the physiological level with agronomic implications.

Glyphosate is a potent herbicide (13). It works by competitive inhibition of the enzyme 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), which catalyzes an essential step in the aromatic amino acid biosynthetic pathway. The measurement of shikimic acid accumulation in response to glyphosate inhibition of EPSPS is a rapid and accurate assay to quantify glyphosate-induced damage in sensitive plants. Pline et al. (14) examined the accumulation of shikimic acid in cotton varieties that were either resistant or susceptible to glyphosate. All tissues of susceptible cotton plants accumulated shikimic acid in response to glyphosate treatment, while glyphosate-resistant plants accumulated much less shimikic acid. The active site of the enzyme EPSPS has been probed using site-directed mutagenesis and inhibitor binding techniques (15). The studies suggest a high degree of structural conservation from bacteria compared to plant EPSPS enzymes.

Previous research has indicated the propensity of horseweed to develop resistance to herbicides. Populations of horseweed resistant to the herbicide paraquat were found in Ontario, Canada (16). These paraquat-resistant populations required doses > 25 times higher than susceptible populations for equivalent control. Horseweed resistant to paraquat (17) and triazines (18) was also documented from collections in Hungary.

MATERIALS AND METHODS

Greenhouse methods. Horseweed plants were collected from two suspected resistant populations in western Tennessee located in Lauderdale county (100 kilometers northeast of Memphis, 35.7N latitude, 89.5W longitude) and from a non-resistant, susceptible horseweed population in Knoxville, TN (36N latitude, 84W latitude). Plants contained within an intact soil core were carefully removed from their native location and transferred to pots (15 cm diameter by 12 cm height) for study in the greenhouse. Each pot contained a single horseweed plant, and was used as an individual experimental unit. The collections were from three distinct populations and were different sizes at the time of collection. The two suspected resistant populations are hereafter denoted as Resistant-East and Resistant-West in the manuscript. Approximate heights at the time of herbicide application were 20 cm for Resistant-East, 10 cm for Resistant-West, and 15 cm for Susceptible. Plants size and plant height were uniform within a given population. Each data point for the greenhouse trials presented in the tables is the numerical mean of five individual experimental units, each consisting of one plant. The study design was constrained by the limited number of glyphosate-resistant plants that were available. The plants had not been sprayed with glyphosate prior to collection.

Plants were allowed to acclimate to greenhouse conditions for two weeks and were watered the evening prior to initiation of the study. Watering was resumed 24 hours after glyphosate application. On May 6th, plants were randomly distributed for two studies and sprayed. The first study was to confirm that these horseweed populations were in fact

resistant to glyphosate. The second study was for shikimate analysis after glyphosate application.

Glyphosate was applied using an enclosed spray booth to prevent movement to non-target plants. Application was made in a water carrier at 190 L/ha applied in two passes (95 L/ha per pass) to provide more complete foliar coverage.

In study one (glyphosate-resistance confirmation), the plants were allowed to grow for 17 d after application of either 0, 0.84, or 3.8 kg ae/ha of glyphosate (commercial formulation of RoundupUltraMax™ was used). A visual evaluation of total plant decline was conducted at 14 DAT. This visual evaluation utilized a 0 to 100 scale, with 0 being no visible effects and 100 being plant death. Shoot fresh weights were obtained by excising each plant at the soil level and weighing on a top-loading balance.

In study two (determination of shikimate accumulation), plants were sprayed as previously described with glyphosate at 0 or 0.84 kg ae/ha. Shoot tissue (top 10 cm of each plant) was harvested 2 and 4 DAT. These sampling periods were chosen to bracket the anticipated time of maximum shikimate accumulation based upon accumulation times reported for soybean (19), tomato (20), and oil seed rape (21). Each plant tissue sample was collected and weighed prior to analysis. Immediately after recording the plant tissue fresh weight, each sample was stored on dry ice and transported to the processing facility. Sample size was 5 plants per population per treatment.

Laboratory methods. Upon receipt of the plant samples (less than 12 hr), the tissue samples were placed into freezer storage (-20C) until processed and analyzed. All

shikimate analyses were completed within 54 days of sample collection. Shikimate is stable for up to 90 d in corn tissue stored at -20C (Massey, unpublished data). Based on these findings, it is anticipated that shikimate will be stable in horseweed tissue when stored at -20C for this period of time.

An extraction procedure similar to one previously reported for corn and soybean (19) was used to analyze the horseweed tissue for shikimate. Frozen horseweed tissue was finely ground in liquid nitrogen using a mortar and pestle. After grinding, the tissue was weighed into 50-mL screw-cap polypropylene centrifuge tubes and 1 M HCl added at a ratio of 5 mL HCl solution per 1 g tissue. The tissue sample sizes ranged from 0.95- to 7.85-g (fresh weight). The samples were placed on an orbital shaker at 1500 RPM for 24 h. For each set of ten samples, a minimum of two untreated blanks and two fresh-fortifications (50 and 500 ppmw shikimate) were prepared. The shikimate (Sigma-Aldrich, St. Louis, MO; 99% purity) fortification solution was prepared in acetonitrile containing 5% water (v/v); the solvents were allowed to evaporate thoroughly in a fume hood before the addition of extraction solution.

Pilot studies indicated that shikimate recovery from horseweed tissue that had been finely ground in liquid N_2 and extracted for 24 h in 1 M HCl were acceptable (Table 1). Recovery of shikimate from horseweed fortified to 50, 500 and 2000 µg shikimate/g and shaken for 24-h averaged 109 ± 21.5 %, 95.0 ± 1.5 %, and 82.5 ± 9.6%, respectively. Moreover, recovery of endogenous shikimate did not change significantly after 24 h shaking (Table 1). Taken together, these results indicated that 24 h shaking with 1 M HCl

was a satisfactory means of extracting shikimate from horseweed. After extraction, each HCl extract was filtered through a Whatman No. 1 filter paper into a graduated cylinder, and the volume of the filtered extract recorded. Next, the pH of the filtered extract was adjusted to 3.0 to 3.3 using saturated NaOH and/or 0.01 N NaOH, as needed. The final volume of the pH-adjusted extract was recorded and returned to the initial extract volume using 0.001 M HCl. The extract 2 mL was diluted with 1.0 mL acetonitrile and passed through a 0.45 micron nylon syringe filter into a chromatography vial. The extract was refrigerated at 4 C until analysis using HPLC.

Analytical method for shikimate. The concentration of shikimate in horseweed tissue was determined by HPLC (19) using an Agilent (Wilmington, DE) series 1100 chromatograph equipped with Chemstation software, auto-injector and photo diode array detector using a detection wavelength of 215 nm. A Phenomenex (Torrance, CA) Luna NH_2 100A column (250 mm* 4.0 mm; 5 micron particle size) was used with an injection volume of 10 µL. The isocratic system used 90/9/1 acetonitrile/deionized water/phosphoric acid and a flow rate of 1.0 mL/min. The total runtime was 20 min with shikimate retention time at 7.4 min. A six-point calibration curve with shikimate concentrations ranging from 3.65 to 52.3 ppm was used to externally quantify shikimate levels in the tissue extracts. The method detection limit for shikimate was approximately 20 ppmw. Representative chromatograms showing extract concentrations of shikimate in horseweed before and after glyphosate treatment are shown in Figure 1. The shikimate

data were analyzed as a completely randomized design using SAS Proc GLM procedure (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Greenhouse studies. This research conclusively confirmed that the suspected glyphosate-resistant horseweed is resistant to glyphosate (Table 2). Visual evaluations 14 DAT indicated less than 15 % control in resistant populations, while the susceptible plants showed 99 % control. However, all resistant plants showed slight phytotoxicity from glyphosate application. The glyphosate-resistant horseweed plant shoot apices turned light green to yellow, the plants were slightly stunted, then resumed normal growth after 5 to 10 days (variable with plants). There were some differences between the two resistant populations. Resistant-East plants were larger at the time of glyphosate application, and they had approximately 45 % growth reduction on fresh weight basis compared with the untreated plants. Resistant-West plants increased in size about 20 % at the low glyphosate rate, or stayed the same size when treated with the higher glyphosate application rate. The resistant populations were contrasted by the susceptible populations that had greater than 80 % decline in plant fresh weight. This small amount of fresh weight plant material was essentially a dead stem that remained from the original plant. These results are in agreement with those of VanGessel, which first reported glyphosate resistant horseweed in Delaware (9).

Laboratory studies. Shikimate recovery from the freshly-fortified control samples was corrected using the appropriate untreated control concentrations. The average

background level of shikimate in untreated horseweed was 69 ± 55 ppmw (n=16) for all untreated horseweed populations and study times. The average recoveries of shikimate from freshly-fortified horseweed tissue were 99 ± 20 % (n=5) at the 50 ppmw level and 86 ± 5 % (n=5) at the 500 ppmw level of fortification.

Shikimate accumulation in glyphosate-resistant and glyphosate-susceptible horseweed. Shikimate accumulated in concentrations significantly greater than background levels after glyphosate treatment in all horseweed populations (Figure 2). There were no significant differences ($\alpha = 0.05$) in shikimate levels among the glyphosate-resistant (i.e., East and West) and glyphosate-susceptible populations 2 and 4 DAT (Figure 2). The two horseweed biotypes differed in the trend over time in shikimate concentration: it decreased from 2 to 4 DAT in the resistant plants, but increased from 2 to 4 DAT in the susceptible plants. One would expect resistant biotypes to have lower pools of shikimate compared to susceptible plants upon herbicide treatment, supposedly at levels close to plants not exposed to glyphosate. The blockage of the EPSPS enzyme is the mechanism of glyphosate activity in plants, so less plant effect would imply less shikimate. Based upon prior studies with glyphosate-tolerant crops (14), the accumulation of shikimate in a resistance population was unexpected.

Taken together with the whole plant bioassays, the shikimate accumulation data indicate that the mechanism of glyphosate resistance in horseweed is not due solely to a single, glyphosate-insensitive EPSPS. If a glyphosate-resistant EPSPS were present, we would not expect to see significant increases in shikimate. While the mechanism of

glyphosate resistance in horseweed is not known, we have several possible hypotheses. Firstly, multiple EPSPS genes encoding various EPSPS isoforms may be present that are responsible for varying levels of inhibition by glyphosate herbicide. Secondly, this glyphosate-resistant horseweed may possess a glyphosate oxidase reductase (GOX)-like enzyme. This scenario is unique in plant science, since no wild, non-transformed plants have been documented to have native GOX genes. The GOX gene was originally derived from non-plant sources and inserted into several plants to increase the selectivity level of those crops to glyphosate (22). The GOX enzyme accelerates the normal degradation of glyphosate into aminomethylphosphonic acid and glyoxylate. Differences in accumulated shikimate levels between 2 and 4 DAT for the resistant and susceptible populations suggest that the resistant plants were able to metabolize accumulated shikimate. This metabolism would support the hypothesis that the biosynthesis of an altered, secondary form of EPSPS enzyme may be induced when the resistant plants are placed under stress by treatment with glyphosate. Specifically, the phenotypic characterizations of glyphosate-resistant horseweed plants post-application and the dynamics of shikimate accumulation, in which shikimate quickly builds up, indicates that glyphosate is initially inhibiting EPSPS, but later the shikimate concentration decreases in glyphosate-resistant plants at 4 DAT and they survive and grow. This is in contrast to the continual increase in shikimate in susceptible plants, which are subsequently killed. An example of this scenario would be how some

herbicide safeners act using a chemical induction mechanism to involve enzymes in herbicide metabolism (23).

A third possible hypothesis deals with the presence of an altered EPSPS. In this scenario, glyphosate competitively binds to EPSPS in the cytosol as well as in the chloroplast. Since natural isoforms of the EPSPS are not over-expressed through genetic manipulation, it is possible that a small pool of altered EPSPS functions normally to deplete the large pool of shikimate that builds up after glyphosate binds to and inhibits the susceptible form of EPSPS. As glyphosate binds to the susceptible form of EPSPS, resistant plants would display altered growth due to a lack of aromatic amino acids. Altered EPSPS would then slowly restore the pathway leading to a depletion of the shikimate pool and continued vegetative development.

Glyphosate-resistant horseweed from Delaware has previously been examined to elucidate the resistance mechanism (24). Initial indications are that glyphosate uptake into the plant and subsequent translocation to the active site were not responsible for the observed resistance. However, enhanced glyphosate metabolism was also not implicated in this preliminary report. A hypothesis of that research group (24) was that an altered form(s) of the EPSPS enzyme was present in glyphosate-resistant horseweed, although the plants retained some susceptible isoforms of the same enzyme. Our results showing a recovery of growth and declining shikimate concentration are consistent with this hypothesis.

The present study confirms glyphosate-resistance in different horseweed populations than previously reported in Delaware (9). The full extent of the occurrence of glyphosate-resistant horseweed in the mid-southern United States is not known, although a preliminary estimate for western Tennessee is 200,000 hectares (unpublished data). To date there are few confirmed locations of the occurrence, but others are suspected.

Our research also presents a series of novel findings, such as the occurrence of shikimate accumulation in both glyphosate-resistant and glyphosate-susceptible horseweed plants. It appears as if the horseweed may either contain a secondary glyphosate-insensitive EPSPS enzyme or contain additional enzymes capable of slowly detoxifying herbicides such as glyphosate. Future research efforts include further studies to determine the molecular mechanism for the observed glyphosate resistance. A molecular analysis for both resistant and other horseweed populations with varying glyphosate susceptibility, focusing on sequence analysis of genes encoding EPSPS and GOX-like proteins will be conducted. These results will be useful in studying the population genetics of the observed resistance, and potential solutions and recommendation for glyphosate resistance management. Additional research conducted under field conditions is currently underway to determine best management practices to control glyphosate-resistant horseweed while maintaining no tillage production practices.

ABBREVIATIONS USED

EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; DAT, Days after treatment with glyphosate; glyphosate oxidase (GOX).

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PART III.

RESPONSE OF SELECTED HORSEWEED [Conyza canadensis (L.) Cronq] POPULATIONS TO GLYPHOSATE[2]

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ABSTRACT

Horseweed (*Conyza canadensis* (L.) Cronq.) seed was collected in Illinois, Indiana, Kentucky, Mississippi, Missouri and Ohio to determine susceptibility of different horseweed biotypes to glyphosate. Horseweed resistant to glyphosate were found in Mississippi, Ohio, and western Tennessee. In a separate experiment examining Tennessee biotypes, a dose response curve demonstrated that four times as much glyphosate was needed to achieve a 50% fresh weight reduction (GR_{50}) in resistant biotypes when compared to a susceptible biotype. Resistant biotypes from Tennessee displayed a GR_{50} of 1.6 kg/ha, as compared to a GR_{50} of 0.4 kg/ha in a susceptible horseweed population. Although growth was reduced, the resistant plants did not completely die and could potentially produce seed. Variation in glyphosate resistance was found among the populations tested.

Keywords. *glyphosate, glyphosate resistance, horseweed, Conyza canadensis, horseweed distribution*

INTRODUCTION

Horseweed [*Conyza canadensis* (L.) Cronq.] is a winter annual or biennial in the Composite family, native to and commonly found throughout North America (1). Horseweed is sometimes referred to as Canada fleabane, mare's-tail, or *Erigeron canadensis* L. Seed dispersal is the only mechanism of horseweed spread and plants are capable of producing over 200,000 small, wind-dispersed seeds per plant in late summer

(2). Plants are ruderal in nature and seeds germinate best in early fall or spring. However, observations indicate that germination can occur throughout the year (1, 3).

The first reported occurrence of glyphosate-resistant horseweed was in Delaware in 2000 (4) followed by reports of similar resistance in west Tennessee (5). No-till crop production has been widely adopted in the mid-Atlantic and mid-South regions, which has favored the establishment and growth of horseweed populations. Horseweed is adapted to periodically plant-free, open, undisturbed soil (6) something often found in no-till crop production systems. Horseweed is less of a problem in tilled fields where fall or spring disking provides control (7). Glyphosate failed to control horseweed in some fields after 3 years of using only glyphosate for weed control in continuous cropping of glyphosate resistant soybeans (4).

The United States Department of Agriculture estimates for crop production indicate that herbicide resistant crop varieties were planted on 80% of soybean hectares and 60% of cotton hectares and 10% of corn hectares for 2003 (8). The use of glyphosate-resistant crops for weed control is common in no-tillage farming practices. A major environmental benefit of no-till systems is reduced soil erosion. In west Tennessee, no-till systems reduce soil erosion by up to 90% (9). In a no-till production system, herbicides are the primary method of weed control due to the lack of soil disturbance by tillage. A no-tillage production system utilizing herbicide resistant crops and a single herbicide, such as glyphosate, could lead to selecting for herbicide resistant weed biotypes with changes at the physiological level that confer resistance to glyphosate (5). Use of glyphosate for

preplant weed control and subsequent postemergence weed control in glyphosate resistant crops has led to the exclusive use of glyphosate on many crop areas, with the result being a decrease in the number of herbicide modes of action on those production areas. Likewise, this system can lead to weed species shifts, where species that were never controlled or were poorly controlled by glyphosate increase in relative abundance. Since horseweed is a winter annual plant that germinates primarily in late winter or early spring in this geographic area, the widespread adoption of no-till systems has greatly increased horseweed's relative abundance.

Glyphosate has a unique mode of action in plants (10, 11, 12). Glyphosate inhibits aromatic amino acid biosynthesis leading to blockage of protein synthesis and secondary metabolite production (10, 13). Glyphosate is a competitive inhibitor of the enzyme 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), which catalyzes an essential step in the aromatic amino acid biosynthetic pathway. EPSPS catalyzes the reaction of shikimate-3-phosphate and phosphenolpyruvate to yield 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate. EPSP is a precursor to chorismate formation, the base molecule for all aromatic amino acid formation.

To adequately discuss herbicidal effects on plants, the ambiguous terminology surrounding plant susceptibility, tolerance, or resistance to herbicides requires clarification. For the purpose of this manuscript the following definitions will be used, and the authors acknowledge that others have expressed slightly different interpretations of these terms. Susceptibility indicates that a plant dies after application of a herbicide at

normal doses (14). Tolerance is the ability of a plant to remain uninjured by herbicide doses normally used to control other plants. Resistance is the ability of a formerly-susceptible plant population to continue to survive herbicide doses above those that were once used to control that original plant population (14).

Essential to the understanding and control of glyphosate-resistant horseweed is determination of the extent of the geographic distribution of glyphosate resistant horseweed in the midsouth and midwest regions of the United States. Previous research has confirmed that horseweed resistance to glyphosate was located in or near Delaware (4) and in a single county in western Tennessee (5). It is suspected that glyphosate resistant horseweed have greatly spread, based on field observations. These anecdotal reports of putative resistance do not confirm resistance, since they do not include susceptible horseweed plants which die from glyphosate application, so they do not provide proof of widespread glyphosate resistance in horseweed. The first research objective was to examine the potential geographic extent of glyphosate resistant horseweed. Information of this nature will allow researchers to determine patterns of distribution and extent of infestation for glyphosate resistant horseweed. Results from this research will also benefit agricultural producers by alerting them to the area of resistance, and will allow them to implement alternative management options for control of this troublesome weed. The second objective of this project was to characterize the sensitivity of horseweed biotypes to glyphosate, resulting in collections of horseweed seeds with varying sensitivity to glyphosate for future study.

MATERIALS AND METHODS

Distribution of glyphosate-resistant horseweed. A horseweed germplasm collection of mature seed heads was conducted in the fall of 2002 at 33 locations in western Tennessee. In the late winter and early spring of 2003, additional collections were made including samples from Illinois, Indiana, Kentucky, Mississippi, Ohio, and the bootheel region of Missouri. Samples were collected from two types of horseweed populations. Firstly, samples were collected where glyphosate resistance would not be expected due to minimal selection pressure. These collection sites included pastures and roadside areas without probable glyphosate application. Secondly, samples were collected from putative glyphosate resistant horseweed locations in no-till production fields. Each location where seed was collected was recorded with global positioning satellite technology. Due to the presentation of a large geographic area some of the collection points on Figure 1 are superimposed on each other, thus you cannot see all the collection points on the figure. For this reason, all the data are presented in tabular form.

Seed were collected from a single seed head and placed directly into large paper bags. The bags were sealed and stored at -4C for <6 months prior to seed processing. Individual seed heads were gently homogenized and the stems and other large plant material was removed. The resulting mixture of seeds and chaff was used in later studies. Care was taken to minimize cross-contamination of seed lots, since the small horseweed seeds were easily moved by wind currents, such as those inside an operating chemical fume hood.

The collected seed were germinated, grown in a greenhouse, and subsequently sprayed with glyphosate to determine sensitivity. To conduct the study, horseweed seed were germinated in styrofoam float trays in soil-less potting media. After germination, horseweed seedlings (1-2 true leaves, 5 mm in height) were transferred to pots containing the same growth media for the duration of the study. Each pot contained a single horseweed plant and was considered to be an individual experimental unit. All treatments were replicated four times and the experiment was conducted twice, so the data presented are the mean of eight observations. Horseweed plants were grown under supplemental metal halide lighting (400 μEinsteins) with 16 h light and 8 h dark periods. Plants were watered twice daily and supplemental fertilizer (MiracleGro™) containing macro and micro nutrients was applied weekly. The time interval from planting seeds to transplanting into cups was 4 wk and the time from transplanting to herbicide application was 5 wk. Plants were watered the evening prior to glyphosate application, and not watered after treatment so as not to wash off the herbicide. Watering was resumed 24 h after glyphosate application. The commercially available potassium salt formulation of glyphosate (Roundup WeatherMax™) was used. Applications of 0, 0.84, and 3.36 kg/ha were applied to 5 cm diameter horseweed rosettes in 190 L/ha of water carrier applied in two passes (95 L/ha per pass) to provide complete coverage. Glyphosate at 0.84 and 3.36 kg/ha represents a 1X (normal) and a 4X dose, respectively. Previous research had indicated that a 4X glyphosate dosage (3.36 kg/ha) was a discriminating application rate to separate resistant from susceptible populations (5). Applications were made in an enclosed spray booth to prevent glyphosate contamination of

non-target plants. Plants were allowed to grow for 21 days after treatment (DAT) to determine glyphosate sensitivity. A visual evaluation of total plant growth decline was conducted at 21 DAT. This evaluation utilized a 0-100 scale, with 0 being no visible effects and 100 being plant death. The visual evaluation incorporated plant size, cholorsis or necrosis, and general plant vigor and robustness. Other data comparing visual evaluations and horseweed fresh weight indicated a high correlation ($R > 0.90$, analysis not shown). Fresh weight determination was problematic due to the variable growth of horseweed plants and also the small amount of plant residue of treated susceptible plants remaining 21 DAT. Visual symptoms clearly elucidated a differential response of various horseweed populations to glyphosate application. Data were subjected to analysis of variance and means were separated by Fisher's Protected LSD (P=0.05). Data are also displayed graphically geo-referenced to the physical location of germplasm collection. For this research, horseweed populations that had <70% injury from glyphosate at 0.84 kg/ha were considered to be resistant, and horseweed populations that had <70% injury from glyphosate at 3.36 kg/ha were denoted as highly resistant. Data were subjected to analysis of variance and means within each herbicide rate were separated using Fisher's Protected LSD test (P=0.05).

Dose response of glyphosate-resistant horseweed. To gain an understanding of the level of glyphosate resistance in Tennessee horseweed biotypes, comparative studies utilized a step-wise rate comparison of glyphosate resistant and susceptible horseweed from normal application rates (0.45 - 0.84 kg/ha) of glyphosate to > 10X rate of 9 kg/ha. Horseweed seed collected from two confirmed resistant biotypes and a confirmed susceptible biotype were

examined (5). The plants were grown in a greenhouse by the previously mentioned methods. Glyphosate rates examined with each horseweed biotype included 0, 0.45, 0.84, 1.25, 1.68, 2.52, 3.36, and 9 kg/ha. Visual evaluations of plant effects were conducted 7, 14, and 21 DAT along with fresh weight determination at 21 DAT. The glyphosate dosages and procedures to determine GR_{50} values are similar to those previously used by VanGessel (4). Data were subjected to analysis of variance and means were separated using Fisher's Protected LSD test (P=0.05).

RESULTS AND DISCUSSION

Distribution of glyphosate resistant-horseweed. Horseweed collected in Mississippi, Ohio, and Tennessee were determined to be glyphosate resistant (Table 1). Analysis of horseweed treated with glyphosate at 3.36 kg/ha indicated population response to glyphosate varied greatly (10-99 %). To account for this population variation, populations which displayed 70% or less control (4 times the LSD) from a 3.36 kg/ha application were classified as highly resistant (HR, Table 1). Horseweed populations where control was 70% or less from 0.84 kg/ha glyphosate was considered resistant (R, Table 1). All other plant responses were defined as susceptible. Plants from the Mississippi location displayed resistance. Plants from one Ohio location were highly resistant, while plants from a separate site in Ohio were susceptible. There was minimal variation in plant response between the 8 experimental units.

In Tennessee, 7 of 32 samples were highly resistant, while 2 other horseweed populations were resistant. The remaining 23 samples were susceptible to both glyphosate application

rates. All horseweed population samples from Illinois, Indiana, Kentucky, and the Missouri bootheel were susceptible to both glyphosate application rates.

Herbicide resistance is dynamic in the ecological plant system. This collection of samples, although covering a wide geographic area, was not exhaustive. It is quite possible that horseweed populations with glyphosate resistance could have been present in adjacent areas at the time of sampling. Additionally, in later years, new horseweed ascensions or introductions could exhibit glyphosate resistance. These data indicated that most of the populations examined in this study were susceptible to glyphosate. While some farmers use no-till production practices, the level of adoption in southern Illinois and western Kentucky is not as extensive as in western Tennessee and in the Delaware region. Greater use of tillage may reduce the incidence of glyphosate resistant horseweed, but this is only a hypothesis (7).

This research suggests that horseweed populations may still be segregating into those that are either glyphosate-resistant or glyphosate-susceptible, based on varying degrees of selection pressure. This research also demonstrated a wide geographical distribution of horseweed that is not controlled by a normal glyphosate application of 0.84 kg/ha (Figure 1). This spread of glyphosate resistant horseweed has been accomplished in a relatively short time period. However, >75% of the sampled horseweed seed lots were still susceptible to glyphosate. The question then arises, are these glyphosate-resistant horseweed from a single source that then spreads, or is glyphosate resistance developing in separate locations as unique events? The exploration of this question will be an area of future study, involving an examination of the physiological and genetic basis for the observed resistance.

Dose response of glyphosate-resistant horseweed. Glyphosate produced some visual symptoms on all horseweed plants from 7 - 21 DAT (Table 2). This plant effect indicated that an active site was still present in even those plants resistant to glyphosate, although effects from 0.84 kg/ha provided only 10-25% control 21 DAT. Glyphosate-susceptible horseweed displayed an increase in control from 7 to 14 and then to 21 DAT, while glyphosate resistant horseweed displayed no change in control from 7 to 21 DAT (Table 2).

Glyphosate at 0.84 kg/ha controlled the susceptible biotype (86%), while the same application rate controlled both resistant biotypes < 20% (Table 2). The susceptible biotype was completely controlled (99%) by glyphosate applications of 1.68 kg/ha or greater. The resistant biotypes required 9 kg/ha glyphosate and 3.36 kg/ha glyphosate or greater to achieve the same control of biotype I and II, respectively. While these application dosages provided statistically similar control, the plants never completely died and thus could possibly continue to grow and produce seed. The production of seed from plants treated with glyphosate has been verified to occur under field conditions (15).

Horseweed fresh weight decreased with increasing glyphosate application rate (Table 2). Susceptible horseweed displayed >70% fresh weight reduction with any glyphosate application and a calculated GR_{50} of 0.4 kg/ha (Figure 2). Resistant biotypes I and II required glyphosate applications of 1.6 kg/ha to achieve 50% fresh weight reduction. Analysis of the GR_{50} of resistant to susceptible populations showed a 4:1 ratio. These results are consistent with previous research by VanGessel (4).

There was no apparent growth reduction of horseweed plants associated with glyphosate resistance. In the absence of glyphosate application, fresh weight of susceptible horseweed (17.2 g) was similar to resistant horseweed (14.5 and 13.2 g). The two types of horseweed plants (resistant and susceptible) looked identical until you sprayed them with glyphosate.

Glyphosate resistance in horseweed and other weeds could have a detrimental impact on current cropping systems in the midsouth and midwest regions of the United States. Results from these studies suggest that resistance is becoming more widespread with resistant biotypes being found in Mississippi, Ohio, and throughout western Tennessee. Special care should be taken to control horseweed with weed management strategies other than glyphosate. It should be noted, however, that greater than 75% of the horseweed seed lots collected were still susceptible to glyphosate. Since glyphosate had activity on most of the horseweed populations, it is possible that other factors could be partially explaining the increase in horseweed occurrence. Environmental conditions such as wet weather, or changes in production systems such as a lack of residual soil-applied herbicides, or a decrease in tillage operations, and other soil factors may be a cause of greater horseweed germination and growth. Future research hopes to elucidate the genetic similarity/dissimilarity of the collected germplasms, possibly to determine if the ascension of glyphosate resistant horseweed is from a single source or from multiple sources.

ACKNOWLEDGMENTS

Appreciation is extended to Pat Brawley and Paul Hahn for their assistance in collecting horseweed samples and conducting this research. Technical advice on horseweed germination and growth conditions provided by Mark VanGessel is also greatly appreciated.

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PART IV

SHIKIMATE ACCUMULATION PROFILES IN GLYPHOSATE-SUSCEPTIBLE AND GLYPHOSATE-RESISTANT HORSEWEED [*Conyza canadensis* (L.) Cronq.][3]

[3] To be submitted to *The Journal of Agriculture and Food Chemistry*.

ABSTRACT

The response of shikimic acid levels in shoot and root tissue of glyphosate-susceptible (GS) and glyphosate-resistant (GR) horseweed biotypes was investigated. Both horseweed biotypes displayed an increase in shikimic acid indicating that 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) remained sensitive to glyphosate. Shikimic acid levels in both shoots and roots of GS horseweed displayed a increasing sigmoidal response to glyphosate, while in GR horseweed shikimic acid levels displayed a log normal peak response with a maximum concentration occurring around 72 hours after treatment (HAT) in both shoot and root tissue. Shikimic acid concentration in GR horseweed began to decrease between 72 and 96 HAT indicating that the shikimic acid pathway resumed at least partial function in the presence of glyphosate. At 168 HAT shikimic acid levels in GS horseweed displayed a 6:1 increase in shoot tissue and a 3:1 increase in root tissue when compared to GR horseweed. This ratio corresponds to previously observed differences in whole plant sensitivity to glyphosate for GS and GR horseweed. These results imply that horseweed resistance to glyphosate is not due to change in the site of herbicide action. The mechanism of resistance appears to be similar to GR ryegrass. Shikimic acid decrease over time could be due to the presence of EPSPS with less sensitivity to glyphosate allowing the pathway to operate, depleting the shikimic acid pool, thus allowing the plant to continue growth.

Keywords. *shikimic acid, shikimate, glyphosate, glyphosate resistance, horseweed, EPSPS.*

INTRODUCTION

Horseweed resistance to the herbicide glyphosate has been documented in Delaware, Tennessee, Mississippi, Ohio, and is putatively reported to exist in Kentucky, Indiana, Maryland, New Jersey, Arkansas, and North Carolina (1, 2, 3, 4, 5). The mechanism of glyphosate resistance in horseweed has been elucidated as a function of limited translocation to meristematic regions (6). This is consistent with findings from Australia for the mechanism of glyphosate resistance in biotypes of rigid ryegrass (*Lolium rigidum*) (6, 7, 8).

Glyphosate has a unique mode of action in plants with activity in the shikimate pathway which occurs in the cytosol and plastids. (6, 8, 9, 10). It inhibits aromatic amino acid biosynthesis, leading to blockage of protein and secondary metabolite production (8, 11). Glyphosate works by competitive inhibition of the enzyme 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), which catalyzes an essential step in the aromatic amino acid biosynthetic pathway. EPSPS catalyzes the reaction of shikimate-3-phosphate and phosphenolpyruvate to yield 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate. EPSP has been demonstrated to be a precursor to chorismate, the base molecule for all aromatic amino acid formation. Measurement of shikimic acid accumulation in response to glyphosate inhibition of EPSPS is an accurate assay to quantify glyphosate-induced damage in sensitive plants (9). Since glyphosate is a potent EPSPS inhibitor, perhaps limitations to glyphosate mobility within a plant can explain glyphosate resistance in plants (6, 7). Only goosegrass (*Elusine indica* L.) in Malaysia has been found to contain a naturally occurring alteration to a EPSPS target site (12).

Results from Mueller et al. (2) provided insight into shikimic acid response two and four days after treatment with glyphosate. Feng and others (6) found a strong correlation between impaired glyphosate translocation and glyphosate resistance in horseweed and proposed reduced phloem loading and reduced EPSPS inhibition as a model to describe glyphosate resistance in horseweed. A more complete time-course of sampling for shikimic acid accumulation in horseweed including examination of root tissue response could provide an interesting whole plant response for the investigation of glyphosate resistance.

The objectives of this research are to determine shikimic acid flux over time after application of glyphosate at the normal field use rate of 0.84 kg ae/ha in the shoot and root of glyphosate-resistant (GR) and glyphosate-susceptible (GS) horseweed populations. Results will provide information about glyphosate resistance at the whole-plant physiological level in horseweed.

MATERIALS AND METHODS

Horseweed seed were collected from a confirmed GR horseweed population in western Tennessee located in Lauderdale county (100 kilometers northeast of Memphis), and from a GS horseweed population in Knoxville, TN (2, 3). These two populations have identical morphology and growth characteristics (data not shown). Horseweed seeds were planted in 30 by 15 by 4 cm styrofoam trays containing soil-less potting media. After emergence, two-leaf horseweed seedlings were transferred to 10 cm diameter styrofoam pots containing soil-less potting media for the duration of the study. Each pot contained a single plant and was used as an individual experimental unit. Plants were grown under supplemental metal halide

lighting (400 μEinsteins) with 16 h light and 8 h dark periods. Plants were watered twice daily and supplemental fertilizer containing macro and micro-nutrients was applied weekly. Plants were watered the evening prior to glyphosate application. Watering was resumed 24 hours after glyphosate application.

Glyphosate was applied at 0 or 0.84 kg ae/ha with water carrier at 190 L/ha using an enclosed spray booth to prevent movement to non-target plants. Plants were harvested 8, 24, 48,72, 96, and 168 hours after treatment (HAT) for shikimic acid analysis. GS plants harvested >168 HAT were mainly necrotic tissue and had insufficient plant material for analysis (data not shown). Horseweed shoot tissue was removed by cutting the stem at the soil-less potting media surface with a scalpel. Root tissue was separated from the potting media and gently washed with water to remove potting media from the roots. Each plant tissue sample was weighed prior to analysis. Immediately after recording the tissue fresh weight, each sample was placed into freezer storage (-20C) until processed and analyzed. All treatments were replicated three times for each sampling interval and the experiment was conducted twice.

Laboratory methods. An extraction procedure similar to one previously reported for horseweed (2) was used to analyze horseweed tissue for shikimate concentration. Frozen horseweed tissue was finely ground in liquid nitrogen using a mortar and pestle. After grinding, the tissue was placed into a 20-mL screw-cap glass scintillation vial and 15 mL of 1 M HCl was added. The samples were placed on an reciprocating shaker at 180 RPM for 16 h. For each set of four samples, a minimum of two untreated blanks and two fortified

standards (50 and 500 ppmw) were prepared. Solvents (pesticide-residue grade) and reagents (reagent-grade) were obtained from Fisher Scientific (Suwanee, GA 30024).

After extraction, each HCl extract was filtered through qualitative filter papaer (Whatman, Clifton, NJ 07014, No. 1 filter paper). A 5.0 mL aliquot of the extract was then adjusted to a pH of 3.0 to 3.3 using 250 uL of saturated NaOH. The sample extract was then diluted with 2.5 mL acetonitrile and passed through a 0.45 um nylon syringe filter. 3.0 mL of this solution was pipeted into a 4.0 mL chromatography vial for analysis. The samples were refrigerated at 4°C until analyzed by HPLC. All samples were analyzed <2 days after extraction.

Analytical method for shikimate. The concentration of shikimic acid in horseweed tissue was determined by Liquid Chromotrography (13) using a Waters (34 Maple Street Milford, MA 01757) chromatograph equipped with a UV detector using a detection wavelength of 215 nm. Data capture was performed using Chemstation software (Agilent Technologies, Foster City, CA 94404). A Phenomenex (Torrance, CA 90501) Luna NH_2 100A column (250 x 4.0 mm; 5 µm particle size) was used with an injection volume of 10 µL. The mobile phase was 90/9/1 (v:v:v) acetonitrile/deionized water/phosphoric acid at a flow rate of 1.0 mL/min. Total runtime was 20 min with a shikimic acid retention time of 8.9 min. The method detection limit for shikimate was approximately 15 ppm in horseweed tissue.

Analysis of variance was conducted for horseweed biotype response to glyphosate. Shikimic acid accumulation data was subjected to regression analysis with Sigma Plot (Systat Software, Point Richmond, CA 94804).

RESULTS AND DISCUSSION

Shikimic acid levels for untreated horseweed shoot and roots were the same for both the GR and GS horseweed biotypes (Figure 1). Therefore, results for shikimic acid levels in untreated horseweed are pooled over both biotypes. No differences were detected in fresh weight of GS or GR shoot or root samples for the duration of the study (data not shown). Background levels of shikimic acid were 37 to 48 and 23 to 34 μg/g fresh weight for untreated horseweed shoot and root, respectively. Horseweed treated with glyphosate displayed differences in shikimic acid levels based on biotype (GS horseweed or GR horseweed) ($P<0.0001$), and sampling time after treatment ($P<0.0001$).

The ratio of shikimic acid levels for treated horseweed indicate dramatic increases compared to untreated tissue (Figure 1). Shikimic acid levels in both shoots and roots of GR and GS horseweed displayed a log normal peak and sigmoidal response to glyphosate, respectively (Figure 1). By 168 HAT shikimic acid levels increased by 192x in shoot and 37x in root tissue of GS horseweed (Table 1). In GR horseweed, shikimic acid levels displayed a log normal peak response to glyphosate with a maximum concentration occurring around 72 HAT in both shoot and root tissue. By this time shikimic acid concentration had increased 47x in shoot tissue and 21x in root tissue. Shikimic acid levels in shoot and root tissue of GR

horseweed decreased after 72 HAT. By 168 HAT shikimic acid levels in GR horseweed had increased by 31x for shoot tissue and 13x for root tissue compared to untreated levels.

Results from this study are interesting for several reasons. Shikimic acid levels in shoot tissue (45x) of GR horseweed increase more rapidly by 48 hours compared to GS horseweed (28x), while shikimic acid concentration in root tissue increase to similar levels by 72 HAT for both GR (21x) and GS (22x) horseweed (Table 2). However, shikimic acid levels begin to decrease by 96 HAT in shoot and root tissue of GR horseweed while shikimic acid levels continued to increase throughout the time course for GS horseweed. These data are consistent with Feng et al. (6) in that these results would be explained by reduced phloem loading of glyphosate in GR horseweed, since shikimic acid levels stop increasing in root tissue 72 HAT. However, shikimic acid concentration in GR horseweed root tissue prior to 72 HAT is similar to the levels found in GS horseweed so it is evident that glyphosate is moving into the phloem in GR horseweed (Table 1). This research indicates that 72 HAT shikimic acid levels stop increasing and begin to decrease in shoot and root tissue of GR horseweed.

Results from this research are also consistent with Mueller et al. (2) in that shikimic acid concentration in GR horseweed begins to decline between 2 and 4 days after treatment while shikimic acid levels continue to increase in GS horseweed for the same time course. Comparison of these results to those of Feng et al. (6) indicates that GR horseweed has three possible mechanisms for glyphosate-resistance: 1) glyphosate is transported into plastids at a reduced rate since shikimic acid levels in GR horseweed peaked at 72 HAT, 2) reduced

loading of glyphosate into phloem tissue for transport to root tissue, shikimic acid concentration peaked at 72 HAT for GR horseweed roots, 3) shikimic acid decrease over time could be due to the presence of three isoforms of EPSPS and possible glyphosate induced amplification of the genes coding for EPSPS (14). Changes in EPSPS may allow the shikimate acid pathway to operate depleting the shikimic acid pool, leading to continued plant growth. The mechanism for glyphosate-resistance in horseweed is different from the genetics that confer glyphosate-resistance to transgenic crops.

This research investigated GS and GR horseweed whole plant response to glyphosate. Glyphosate is active in GR horseweed as evidenced by shikimic acid increase in both shoot and root tissue. The mechanism of resistance appears to be similar to GR ryegrass (6, 7). Eventual shikimic acid decrease over time (after 72 HAT) could be due to the presence of EPSPS with less sensitivity to glyphosate allowing the pathway to operate, depleting the shikimic acid pool, thus plant growth continues. In these studies, the use of agronomically relevant glyphosate doses represents what is actually happening in field production practices. Sub-lethal doses of glyphosate are practical for some research activities, but it is plausible that a plant treated with a sub-lethal dose may respond differently than a plant treated with agronomically relevant dose. Research on horseweed has indicated that glyphosate stunts GR plants, but alternative management strategies are needed (1, 2, 3, 4, 6).

Plant tissue analysis of shikimic acid concentration after treatment with glyphosate is a excellent indicator of glyphosate-resistance at the whole-plant level. Glyphosate activity on susceptible plants limits full dose studies to a 7 to 10 day time course which in the case of

horseweed was adequate to determine the response in the shikimic acid pathway. Future studies will focus on more rapid plant tissue preparation for shikimic acid analysis.

ABBREVIATIONS USED

ae, acid equivelant; EPSP, 5-enolpyruvylshikimate-3-phosphate; EPSPS, 5-enol-pyruvyl-shikimate-3-phosphate synthase; GR, glyphosate-resistant; GS, glyphosate-susceptible; HAT, hours after treatment

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APPENDICES

APPENDIX A

FIGURES

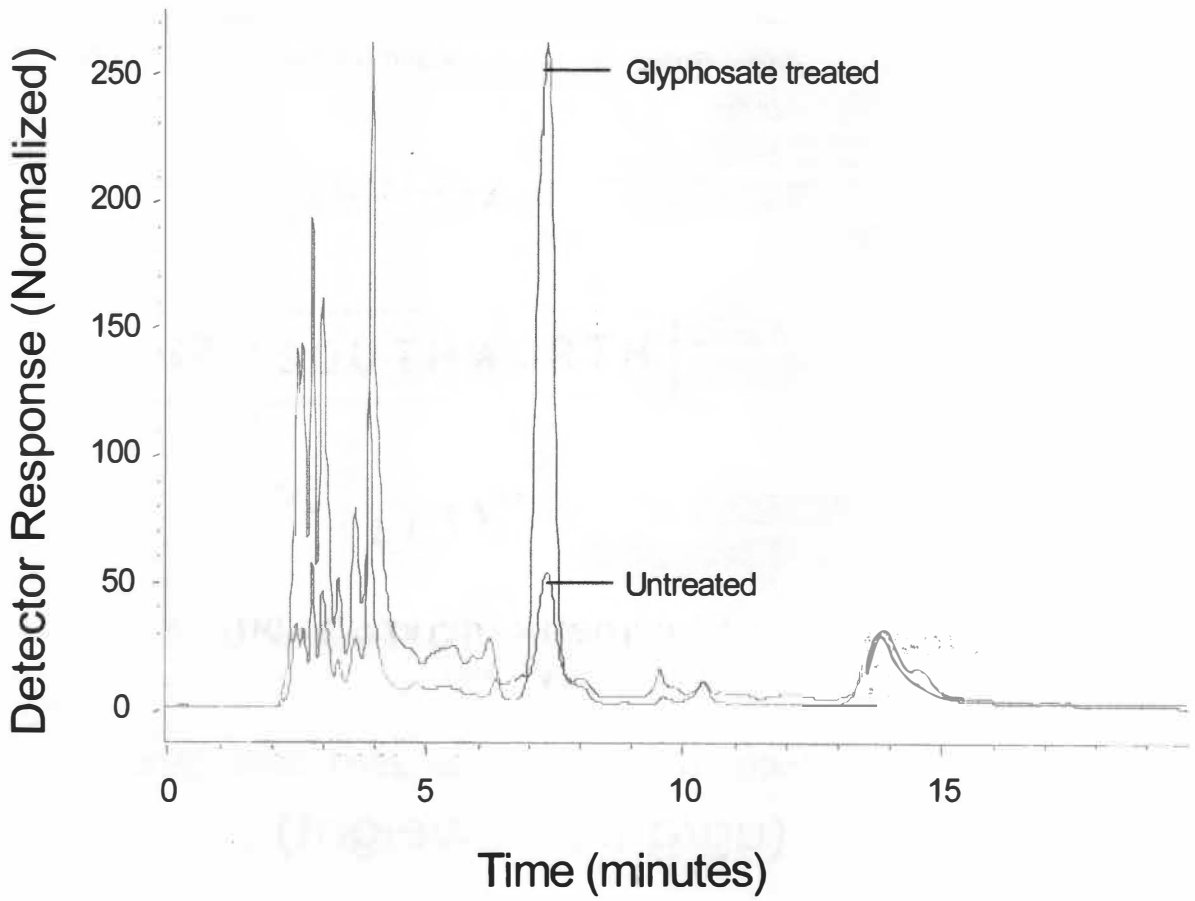

Figure 1. Representative chromatograms for shikimate accumulation and quantification in horseweed two days after glyphosate treatment.

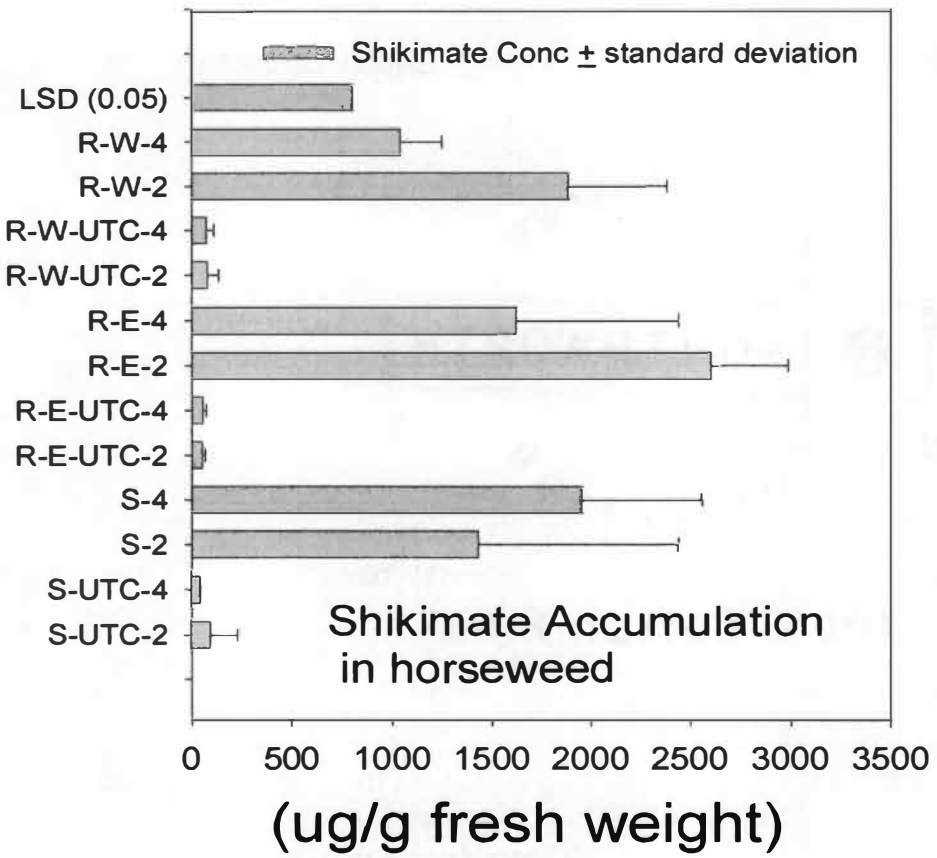

Figure 2. Accumulation of endogenous shikimate in two (denoted East and West) glyphosate-resistant (R) and glyphosate-susceptible (S) horseweed populations determined at 2 and 4 d after glyphosate treatment. Data from Untreated Control (UTC) plants also shown.

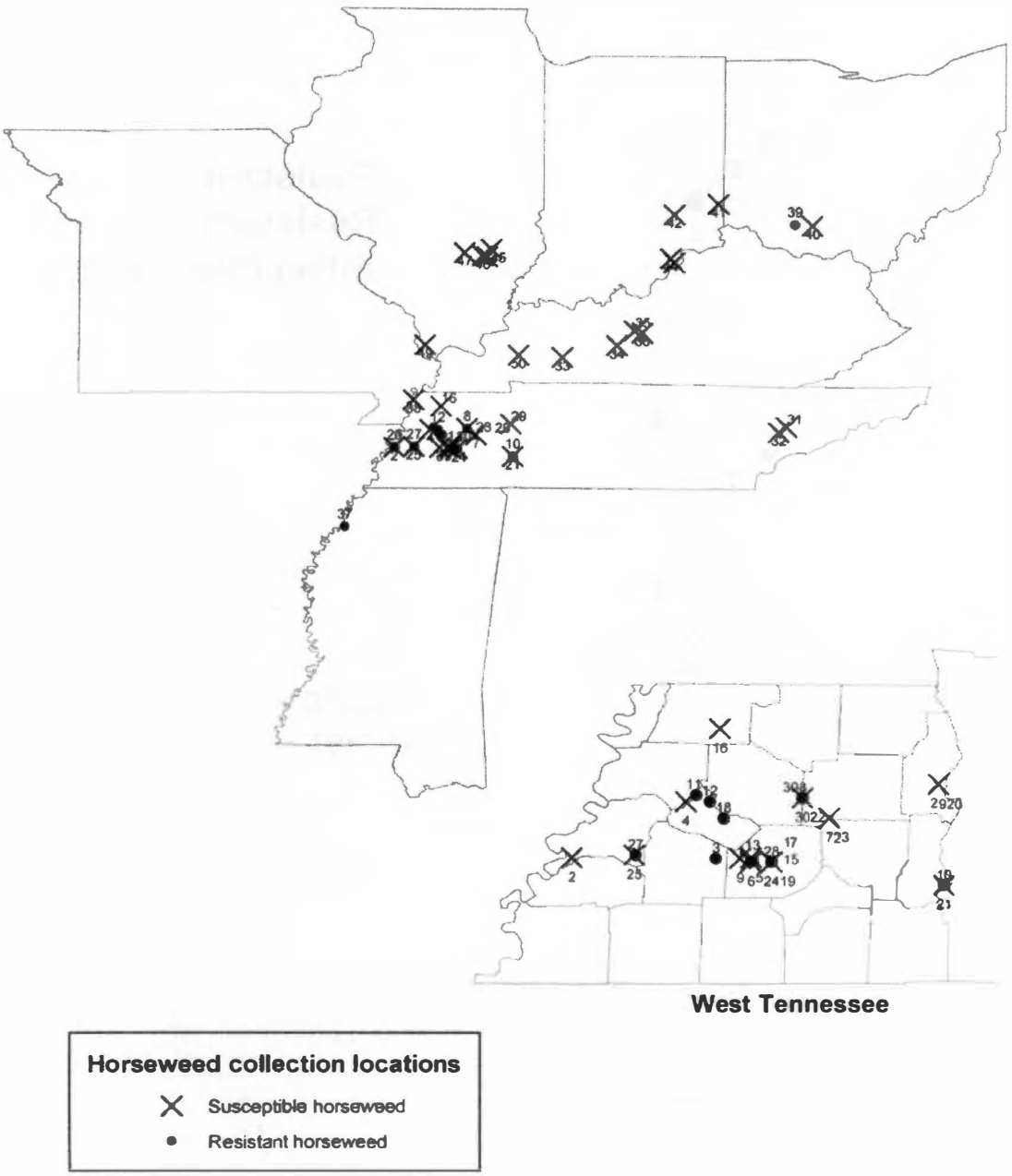

Figure 3. Location of horseweed germplasm collection and response to glyphosate (0.84 kg ae/ha or 3.36 kg ae/ha).

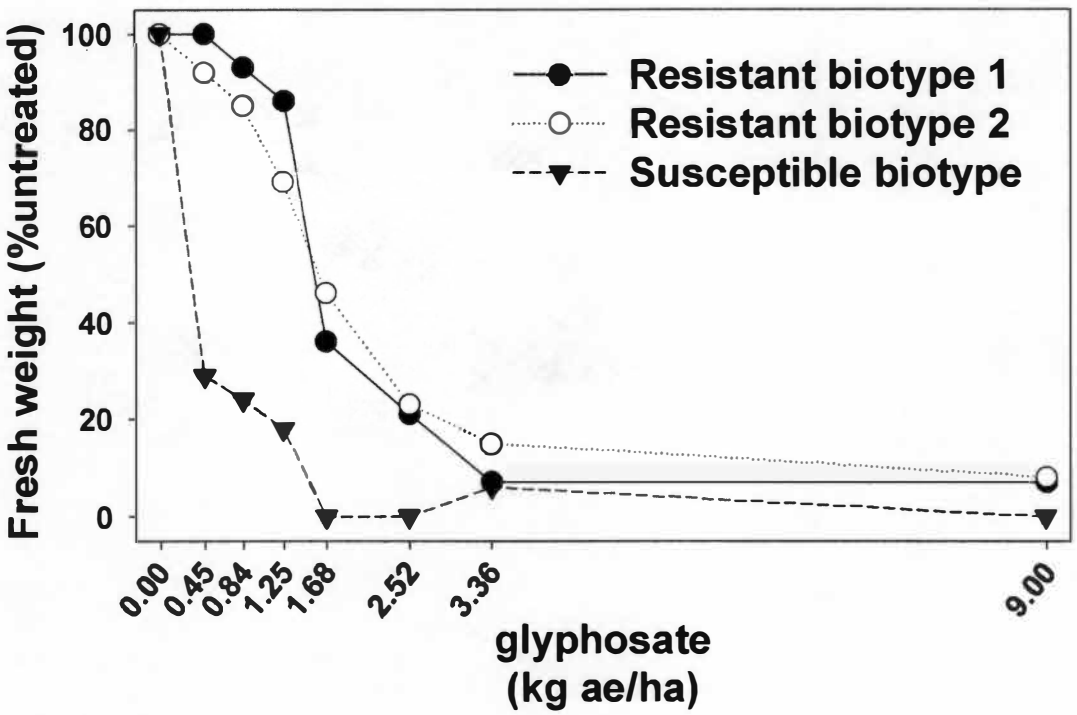

Figure 4. Horseweed biotype response to increasing doses of glyphosate.

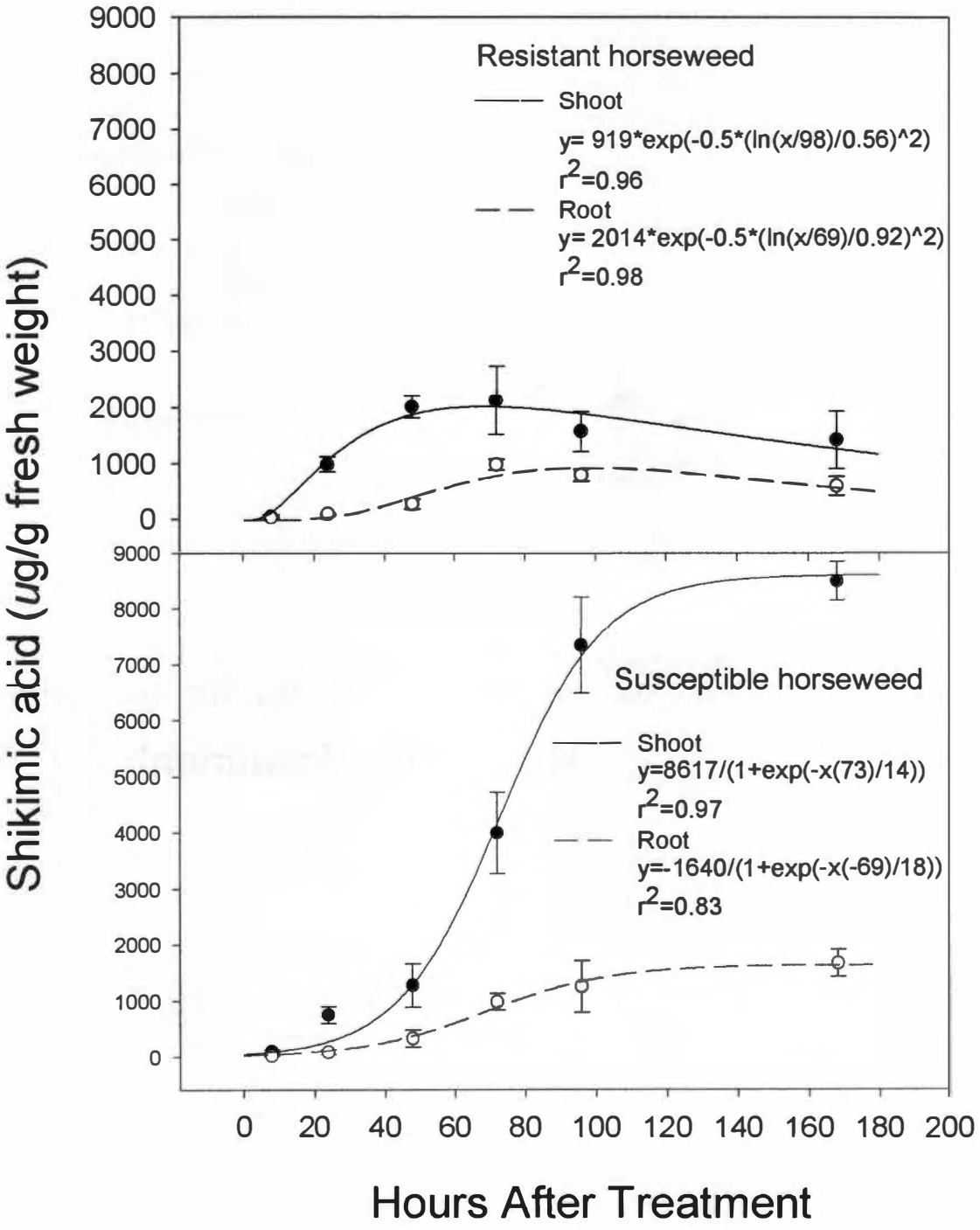

Figure 5. Shikimic acid response in horseweed over time to treatment with glyphosate (0.84 kg ae/ha).

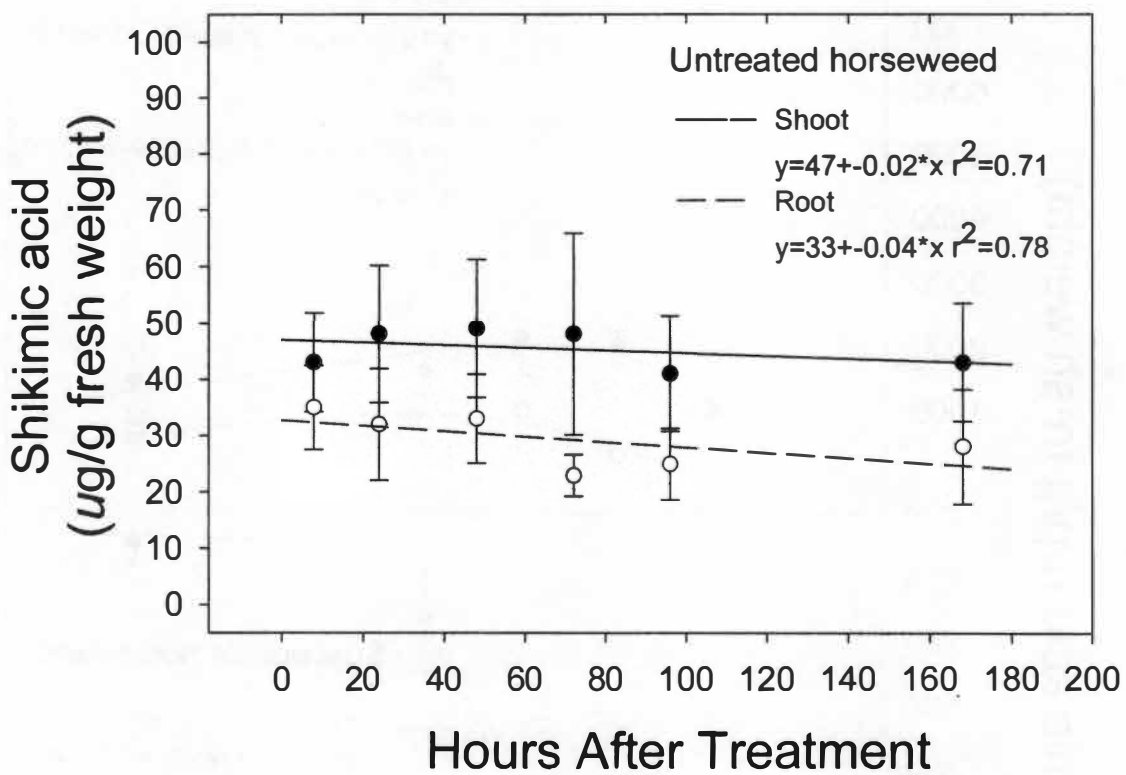

Figure 5. Continued.

APPENDIX B

TABLES

Table 1. Recovery of freshly-fortified and endogenous shikimate from horseweed tissue using 1 M HCl as a function of extraction time.[a]

Shikimate Treatment	Extraction Time(h)	Average Recovery	n
50 μg/g[b] Freshly-Fortified shikimate	24	108.7 ± 21.5 %	2
	48	98.9 ± 14.7 %	2
	72	86.6 ± 15.5 %	2
500 μg/g[b] Freshly-Fortified shikimate	24	95.0 ± 1.5 %	3
	48	94.9 ± 3.0 %	3
	72	84.1 ± 5.4 %	3
2000 μg/g[b] Freshly-Fortified shikimate	24	82.5 ± 9.6 %	3
	48	71.5 ± 3.1 %	3
	72	71.2 ± 2.5 %	3
Endogenous[c] shikimate	24	5807 ± 129 μg/g	3
	48	5964 ± 348 μg/g	3
	72	5854 ± 562 μg/g	3

[a] Extraction time on orbital shaker at 1500 rpm using 5 mL 1 M HCl per g tissue.

[b] Applied to 2-g of untreated, field-grown tissue finely ground in liquid N_2; recovery results are corrected for background shikimate concentrations which ranged from 21 to 85 ppmw.

[c] Endogenous levels of accumulated shikimate in horseweed 3 DAT with 1.9 kg ae/ha glyphosate applied as Roundup Ultramax™ herbicide.

Table 2. Growth reduction and control of horseweed biotypes treated with glyphosate.

Horseweed biotype	Glyphosate dosage	Control at 14 d	Fresh Weight at 17 d	Fresh Weight % of untreated
	kg ae/ha	%	g	%
Resistant-East	0	0	14.91	100
Resistant-East	0.84	4	9.10	61
Resistant-East	3.8	6	7.91	53
Resistant -West	0	0	5.79	100
Resistant-West	0.84	6	7.03	120
Resistant-West	3.8	14	5.54	96
Susceptible	0	0	11.9	100
Susceptible	0.84	99	1.94	16
Susceptible	3.8	99	1.53	13
LSD (0.05)		4.4	2.1	

Table 3. Geographic location and response of horseweed to glyphosate.

Sample	State	Latitude	Longitude	Glyphosate rate (kg ae/ha)			Sensitivity to glyphosate[a]
				0	0.84	3.36	
				% Control			
1	TN	35.62	-89.95	0	13	92	R
2	TN	35.64	-89.82	0	91	97	S
3	TN	35.63	-89.12	0	55	93	R
4	TN	35.92	-89.27	0	99	99	S
5	TN	35.64	-89.95	0	95	99	S
6	TN	35.62	-89.95	0	99	99	S
7	TN	35.83	-89.57	0	99	99	S
8	TN	35.93	-89.70	0	6	32	HR
9	TN	35.63	-89.03	0	69	89	R
10	TN	35.50	-88.01	0	10	10	HR
11	TN	35.95	-89.21	0	30	70	HR
12	TN	35.92	-89.15	0	12	12	HR
13	TN	35.62	-89.85	0	99	99	S
14	TN	35.62	-89.85	0	99	99	S
15	TN	35.62	-89.85	0	99	99	S
16	TN	36.60	-89.10	0	99	99	S
17	TN	35.62	-89.85	0	99	99	S
18	TN	35.83	-89.08	0	31	70	HR
19	TN	35.62	-89.85	0	99	99	S
20	TN	36.60	-88.03	0	99	99	S

Table 3. Continued.

Sample	State	Latitude	Longitude	Glyphosate rate (kg ae/ha)			Sensitivity to glyphosate[a]
				0	0.84	3.36	
				% Control			
21	TN	35.50	-88.01	0	99	99	S
22	TN	35.93	-89.70	0	88	99	S
23	TN	35.83	-88.57	0	94	99	S
24	TN	35.62	-89.85	0	99	99	S
25	TN	35.65	-89.52	0	99	99	S
26	TN	35.64	-89.82	0	7	66	HR
27	TN	35.65	-89.51	0	14	31	HR
28	TN	35.62	-89.85	0	25	55	HR
29	TN	36.60	-88.03	0	94	99	S
30	TN	35.93	-89.70	0	93	99	S
31	TN	35.97	-83.85	0	99	99	S
32	TN	35.88	-83.97	0	99	99	S
33	KY	36.83	-87.25	0	99	99	S
34	KY	37.23	-86.42	0	99	99	S
35	KY	37.47	-86.15	0	99	99	S
36	KY	37.43	-86.03	0	99	99	S
37	MS	34.40	-90.57	0	26	90	R
38	MO	36.38	-89.52	0	99	99	S
39	OH	39.13	-84.73	0	3	33	HR
40	OH	39.12	-83.47	0	99	99	S

Table 3. Continued.

Sample	State	Latitude	Longitude	Glyphosate rate (kg ae/ha)			Sensitivity to glyphosate[a]
				0	0.84	3.36	
				% Control			
41	IN	39.45	-85.88	0	99	99	S
42	IN	39.28	-85.55	0	99	99	S
43	IN	38.53	-85.55	0	99	99	S
44	IN	38.60	-86.60	0	99	99	S
45	IL	38.73	-88.33	0	99	99	S
46	IL	38.62	-88.40	0	99	99	S
47	IL	38.38	-88.75	0	99	99	S
48	IL	38.63	-89.47	0	99	99	S
49	IL	36.90	-89.35	0	99	99	S
50	IL	37.08	-87.92	0	99	99	S
LSD (0.05)					5	6	

[a] S = susceptible biotype; R = resistant to glyphosate at 0.84kg/ha (70% or less control), but susceptible to glyphosate at 3.36kg/ha; HR = resistant to 3.36 glyphosate dose (70% or less control).

Table 4. Horseweed biotype control as effected by increasing glyphosate doses under greenhouse conditions.

Horseweed biotype	Glyphosate rate	Control			Fresh weight (21DAT)	Fresh weight as % of untreated
		%				
	kg ae/ha	7 DAT[a]	14 DAT	21 DAT	g	%
Susceptible	0	0 m[b]	0 k	0 j	17.2 a	100
	0.45	40 ghi	40 fgh	74 abc	5.1 efgh	29
	0.84	58 def	63 de	88 a	4.6 efg	24
	1.25	60 cde	92 ab	90 a	3.2 fghij	18
	1.68	63 cde	98 a	99 a	0.2 j	0
	2.52	83 ab	99 a	99 a	0.3 j	0
	3.36	85 ab	95 ab	99 a	1.1 ij	6
	9	90 a	98 a	99 a	0.2 j	0
Resistant 1	0	0 m	0 k	0 j	14.5 ab	100
	0.45	10 lm	8 jk	11 hij	14.3 ab	100
	0.84	20 jkl	13 ijk	11 hij	13.6 ab	93
	1.25	28 ijk	18 ij	30 fghi	12.8 bc	86
	1.68	40 ghi	40 fgh	43 defg	5.2 efgh	36
	2.52	58 def	45 fg	59 cdef	3.7 fghij	21
	3.36	73 bc	58 def	75 bcde	1.2 j	7
	9	80 ab	80 bc	80 ab	1.4 j	7
Resistant 2	0	0 m	0 k	0 j	13.2 abc	100
	0.45	13 lm	13 ijk	20 ghij	12.5 bc	92

Table 4. Continued.

Horseweed biotype	Glyphosate rate	Control %			Fresh weight (21DAT)	Fresh weight as % of untreated
	kg ae/ha	7 DAT[a]	14 DAT	21 DAT	g	%
	0.84	23 jkl	14 ijk	24 fghij	11.4 bcd	85
	1.25	33 hij	15 ijk	25 fghij	9.6 cde	69
	1.68	50 efg	44 fg	50 cdef	6.5 efg	46
	2.52	53 defg	50 efg	63 bcd	3.2 fghij	23
	3.36	60 cd	69 cd	75 abc	2.4 ghij	15
	9	73 bc	91 ab	89 a	1.6 j	8
LSD (0.05)		14	16	26	4	

[a] DAT = Days after treatment.

[b] means within a column followed by the same letter are not different according to Fisher's Protcted LSD test at P = 0.05.

Table 5. Shikimic acid increase in glyphosate (0.84 kg ae/ha) treated horseweed compared to untreated horseweed.

	Hours after treatment					
Tissue type	8	24	48	72	96	168
	Ratio of shikimic acid (treated:untreated)					
GS[a] shoot	1.5	16	28	90	166	192
GS root	0.0	1.5	7	22	28	37
GR[b] shoot	0.3	21	45	47	35	31
GR root	0.3	1.4	5.6	21	17	13

[a]GS = glyphosate-susceptible.

[b]GR = glyphosate-resistant.

VITA

Christopher L. Main was born April 10, 1976, in Hillsboro, OH. He is the son of Mr. and Mrs. Gary Main. He attended Hillsboro High School and graduated in June 1994. He entered the University of Tennessee in August of 1994 and received a Bachelor of Science in Agriculture, majoring in plant and soil science. Upon graduation, he accepted the position of Graduate Research Assistant in the graduate program at the University of Florida and was awarded a Master of Science in agronomy with a concentration in weed science in 2001. Chris returned to the University of Tennessee in 2001 to pursue a Ph. D. in Plants, Soils, and Insects. Chris is the author of 11 published manuscripts. He has made presentations at the Weed Science Society of America, the Southern Weed Science Society, the Florida Weed Science Society, the Tennessee Agricultural Production Assocation, and the Milan No-Till Field Day. Chris is married to the former Ms. Shelly Hughes of Germantown, TN and is father to one son, Christopher 'Hayden' Main. Chris enjoys spending time with his family, playing golf, and relaxing with a good book. Following completion of his Ph.D. degree, he plans to continue in academia in the area of weed science.